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Genetics

"Mutagenesis" by peptide aptamers identifies genetic network members and pathway connections

(genetic analysis/protein interactions/combinatorial peptide libraries)

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ABSTRACT **We selected peptide aptamers from combinatorial libraries that disrupted cell-cycle arrest caused by mating pheromone in yeast. We used these aptamers as baits in two-hybrid hunts to identify genes involved in cell-cycle arrest. These experiments identified genes known to function in the pathway, as well as a protein kinase, the CBK1 product, whose function was not known. We used a modified two-hybrid system to identify specific interactions disrupted by these aptamers. These experiments demonstrate a means to perform "genetics" on the protein complement of a cell without altering its genetic material. Peptide aptamers can be identified that disrupt a process. These aptamers can then be used as affinity reagents to identify individual proteins and protein interactions needed for the process. Forward genetic analysis with peptide aptamer "mutagens" should be particularly useful in elucidating genetic networks in organisms and processes for which classical genetics is not feasible.**

In classical forward genetics, an investigator isolates organisms that display altered phenotypes and identifies the mutant genes that cause them. Despite its power, forward genetics is often difficult. Many interesting organisms are diploid. In diploids, identification of genes involved in a process typically requires large-scale mutagenesis and identification of recessive phenotypes in F_2 progeny. Recently, the ability to manipulate individual genes has driven the development of reverse genetics, in which the function of genes is inferred from the phenotypes that arise from their mutation. In diploids, reverse genetics also typically requires generation of homozygotes in the mutated gene. To circumvent this requirement, a number of dominant "reverse genetic" methods to inactivate gene function have been devised, including inhibition by drugs (reviewed in ref. 1), expression of dominant-negative proteins (2), injection of antibodies (3), expression of antisense RNAs (4), expression of nucleic acid aptamers (5), and expression of peptide aptamers (6). These methods inactivate the gene product but do not alter the genetic material.

Peptide aptamers from combinatorial libraries can be dominant inhibitors of gene function. As we define them, peptide aptamers are proteins that contain a conformationally constrained peptide region of variable sequence displayed from a scaffold. We and others have used two-hybrid systems to select aptamers based on *Escherichia coli* thioredoxin (TrxA) that recognize specific proteins and allelic variants with K_ds and half-inhibitory concentrations from 1×10^{-8} to 5×10^{-11} M. We have selected aptamers against Cdk2 (6), Ras (ref. 7; C. W. Xu, Z. Luo, and R.B., unpublished data), E2F (8), and HIV-1 Rev (9). In mammalian cells (ref. 10; C. W. Xu, Z. Luo, and R.B., unpublished data) and in *Drosophila melanogaster* (11), such aptamers function as dominant reverse genetic agents.

Here, we used peptide aptamers for forward genetic analysis. We used aptamers as "mutagens" to identify proteins and protein interactions needed for a complex phenotype. When exposed to α-factor, MATa *Saccharomyces cerevisiae* stop cell cycle progression in mid-G_1 and induce transcription of genes needed to mate (reviewed in ref. 12). We selected peptide aptamers that overcame the cell-cycle arrest. We used interaction mating (13) and partial-genome two-hybrid interactor hunts (14) to identify proteins targeted by these aptamers. Our experiments identified known proteins involved in this response. These experiments also identified a protein, Cbk1, not previously known to function in this response. We used a modified interaction trap two-hybrid system to detect specific protein interactions disrupted by the aptamers. These results demonstrate the power of using peptide aptamers to produce a phenotype and to identify proteins and protein interactions involved in it. We expect this approach will aid analysis of processes in which classical transmission genetics is impractical.

MATERIALS AND METHODS

Strains, Conventional Plasmids, and Libraries. *Strains.* HCY5, a MATa/MATa, trp1/trp1, bar1::LEU2/bar1::LEU2 leu2/leu2 ura3/ura3, ade2/ADE2, his3/HIS3, his4–917Δ/his4, can1/can1, lys2–173R2/LYS2 diploid, was used for the α-factor resistance selections. HCY4, MATa lys2 trp1 ura3 leu2 can1 bar1::LEU2, is a precursor of HCY5. These strains were made by Chertkov and Brent and are described in detail at www.molsci.org. EGY40, MATα leu2 his3 trp1 ura3; EGY42, MATa leu2 his3 trp1 ura3; EGY48, MATα his3 trp1 ura3–52 leu2::LexA6op-LEU2 have been described (14, 15). IH2290, MATa leu2 his4 trp1 ura3–52 met1 bar1 fus1::fus1-LacZ::URA3, and IH2272, MATa HMRa HMLa ura3 leu2 trp1 ade2 met1 bar1–1 were gifts from I. Herskowitz (Univ. of California, San Francisco).

Plasmids. All plasmids directed synthesis of chimeric proteins that contained either the full-length named protein or named fragment. Their construction is detailed at www.molsci.org. Preys were derivatives of pJG4–5 (14). pYBS146 (Ste5 residue 24-Cterm); pYEE129 (Ste7), pYBS348 (Ste11); pYBS1 (Fus3); pYBS327 (Kss1)(16) were gifts of E. Elion (Harvard Medical School, Boston). We constructed pJG4–5-Gpa1, pJG4–5-Ste4, pJG4–5-Ste18, pJG4–5-Ste50, pJG4–5-Ste12, pJG4–5-Cln3, pJG4–5-Cln2, and pJG4–5-Cbk1, pJG4–5-Cdc28, pJG4–5-Far1, pJG4–5-Ste20 and pJG4–5-Cln1 by introducing PCR products generated from EGY48 genomic DNA into pJG4–5. To map portions of the target proteins recognized by the aptamers, we used preys pYBS308 and pYBS214 (gifts of E. Elion) that, respectively, expressed Ste5 residues 241–336 and Ste5 residues 24–143 and 309–917 (16).

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To map aptamer targets, we also constructed pJG4–5Ste5Δ1, pJG4–5Ste5Δ2, pJG4–5Ste5Δ3, which respectively expressed Ste5 residues 24–336, 24–586, and 336–586, and pJG4–5-Ste50Δ2, which directed the synthesis of Ste50 residues 68–118, by PCR isolation of the coding sequences from EGY48 DNA, followed by recombination in yeast essentially as described in Ma *et al.* (17). We constructed pJG4–5-Ste11Δ1, pJG4–5-Ste11Δ2, and pJG4–5-Ste11Δ3, which respectively express Ste11 amino acids 1–85, 85–137, and 118–738, and pJG4–5-Ste50Δ1, and pJG4–5-Ste50Δ3, which respectively expressed Ste50 residues 1–68 and 118–346, by introducing PCR products from EGY48 DNA into pJG4–5. We assayed interactions essentially as described in Finley and Brent (13).

Baits were derivatives of pEG202 (14). pYBS325 (Ste7) and pYBS345 (Ste11) (16), were gifts from E. Elion. We constructed pEG202-Fus3 by using the Fus3 gene from pYBS171. We constructed pEG202-Ste50, pEG202-Cbk1, pEG202-Ste20, and pEG202-Ste4 by cloning PCR products generated from EGY48 genomic DNA into pEG202. For the pEG202-C1 to pEG202-N5 series of aptamer baits, we generated PCR products that contained the ORFs of the selected aptamers in aptamer-expression plasmids pJM-2 and pJM-3 (see below) and cloned them into pEG202. To assay portions of the target proteins recognized by aptamers, we used baits that expressed aptamers C1, C4, and N3 and Ste5, Ste50, and Ste11.

Libraries. Peptide aptamer libraries in pJM-2 and pJM-3 were constructed by John McCoy exactly as described for the aptamer interaction library in Colas *et al.* (6). pJM-2 expresses thioredoxin aptamers that contain an amino-terminal hemagglutinin epitope tag, so that their amino-terminal moiety is MYPYDVPDYA. Aptamers expressed by pJM-3 are identical except that they contain an SV40 nuclear localization signal amino terminal to the epitope tag, so that their amino-terminal moiety is MGAPPKKKRKVAYPYDVPDYA. The pJM-2 and pJM-3 libraries have 3.6×10^8 and 6.6×10^8 members, respectively. The pJG4–5 partial coverage yeast library was a gift of P. Watt (Telethion Institute for Child Health Research, West Perth, Australia) (18).

Selection of Aptamers that Confer α-Factor Resistance. We transformed the nonlocalized (pJM-2 derived) and nuclear (pJM-3 derived) libraries into HCY5 (19). We obtained 8.4×10^6 nonlocalized library and 7.3×10^6 nuclear library yeast transformants on Trp$^-$ glucose plates. We grew 17 equivalents of the nonlocalized library and 13 equivalents of the nuclear library for 4 hours in Trp$^-$ galactose media, and then plated these cultures onto Trp$^-$ galactose plates that contained 1 μM α-factor. After 4 days of growth at 30°C, 651 ''nonlocalized'' and 151 ''nuclear'' colonies appeared. Of these, 202 ''nonlocalized'' and 65 ''nuclear'' colonies showed galactose-dependent growth on Trp$^-$ α-factor plates. We recovered the plasmids for 69 ''nonlocalized'' and 28 ''nuclear'' isolates and reintroduced them into HCY5 to reconfirm the aptamer dependent phenotype. Galactose-dependent growth on 1 μM α-factor Trp$^-$ plates was conferred by 88% of the nonlocalized and 96% of the nuclear peptide aptamers. We sequenced 60 of these aptamers with an ABI automated sequencer; this sequencing revealed 20 unique nonlocalized and 18 nuclear peptide aptamers. We arbitrarily chose 12 of these 38 aptamers for further characterization.

We measured the penetrance of α-factor resistance of these aptamers by growing overnight cultures of HCY5 that either contained the peptide aptamer (pJM-C1 to pJM-N5 series) or pJG4–4, a control plasmid lacking the aptamer (14) at 30°C in Trp$^-$ galactose liquid media. We plated ≈100 cells (as estimated from OD_{600}) in triplicate from these cultures onto Trp$^-$ galactose plates that either did or did not contain 50 nM α-factor. We calculated penetrance by colony number after 4 days at 30°C on galactose medium with α-factor compared with that on galactose medium without it. Penetrance ranged from 50% to 97%.

Identification of Targeted Proteins. To roughly map aptamer function within the pheromone-response pathway, we first monitored α-factor induction of the *FUS1-lacZ* gene in IH2290. We introduced aptamer-expressing plasmids (pJM-C1 to pJM-N5 series) or pJG4–4 into IH2290 and grew transformants overnight at 30°C in Trp$^-$ galactose medium. The next day, we diluted these cultures to an OD_{600} of 0.2 and grow them to an OD_{600} of 0.6. We added α-factor to 0.5 μM and incubated the cultures for another hour. We assayed β-galactosidase in these cultures as in Stern *et al.* (20). We next determined whether these aptamers might cause α-factor resistance by derepressing the HMLα locus (21–23) in HCY4 and HCY5. We tested the derepression idea by introducing aptamer-expression plasmids into IH2272, in which HMLa replaces HMLα. We grew overnight cultures of each transformant at 30°C in Trp$^-$ galactose medium. We plated ≈1,000 cells (as estimated from OD_{600}) from these cultures on Trp$^-$ galactose plates that contained or lacked 50 nM α-factor and counted colonies after 3 days at 30°C.

We identified aptamer targets in two ways. First, we tested the aptamers against panels of preys (13). We made bait plasmids that expressed different aptamers as above and introduced them into EGY48. We used preys that expressed known proteins in the α-factor pathway, and introduced them into EGY42 that contained the LexAop-lacZ reporter pSH18–34. We mated these strains and plated exconjugates onto interaction detection plates. For interactions that gave strong blue color on Ura$^-$Trp$^-$His$^-$ galactose/5-bromo-4-chloro-3-indolyl β-D-galactoside medium, we performed liquid β-galactosidase assays.

Second, we identified potential targets by using aptamers as baits in two-hybrid interactor hunts. We chose as baits those aptamers that showed the lowest activation of the LexAop-LEU2 and LexAop-lacZ reporters. We introduced the yeast interaction library into EGY48 that carried aptamer baits and pSH18–34. For each bait strain, we obtained the following numbers of transformants: C2, 2.7×10^6; C6, 1×10^6; N1, 1×10^6; N3, 8.1×10^6; and N5, 2.7×10^6. We selected and characterized interactors as described in Gyuris *et al.* (14). We tested interactors by deleting the genes encoding them in HCY4 and measuring the resistance of these strains to α-factor. We made these deletions by PCR disruption essentially as described (ref. 24; detailed at www.molsci.org), and made strains that lacked the following genes: A1, PBP2, ULA1, PDC6, ADE3, DBI56, ERR1, MSH6, CBK1, MUP3, CHS1, PBP2, SUN4, YDR005C, UBP2, and YDR104C. To measure resistance of the deletions (e.g., HCY4 cbk1Δ) to α-factor, we grew overnight cultures of the deletion and of HCY4 that contained pJG4–4 or AptC6 (see below) in Trp$^-$ galactose medium at 30°C. We plated ≈1,000 cells (as estimated from OD_{600}) on Trp$^-$ galactose plates at 30°C that contained 0, 25, 50, and 100 nM α-factor and counted colonies after 4 days.

Identification of Targeted Interactions. We constructed aptamer expression plasmids pRG1-C1, pRG1-C4, and pRG1-N3 by transferring the GAL1 promoter and DNA that encoded the aptamer-coding sequences, any fused moieties, and the ADH1 terminator from the pJM-2 and pJM-3 library plasmids that expressed them to pGAH-1, which contains a 2 μ origin and LEU2 marker (O. Hobert, unpublished data). We constructed low-level bait expression plasmids (pRG2–1 series) by introducing the ADH1 promoter and terminator from pJK315 (LEU2, CEN-ARS vector with the ADH1 promoter-terminator cassette in pRS315; J. S. Kamens and R.B., unpublished data) into pRS313, (HIS3, CEN-ARS) (25). We cut pRG2–1 with *Sph*I, which cleaves within the ADH1 promoter and terminator, and dephosphorylated the linearized vector. We cut pEG202-Ste50 and pEG202-Ste11 with *Sph*I and from each isolated the fragment that contained 3′ 350 bp of the ADH promoter, the LexA fusion coding sequence, and the 5′ 700 bp of the ADH1 terminator. We ligated this fragment into

Table 1. Peptide aptamers

Aptamer	Variable Region
C1	EWCGP**CESCQILFLQFRCLRFCRRM**GPCKM
C2	EWCGP**LHSSELECRISGFLSVSMLL**GPCKM
C3	EWCGP**RLRAQRRDWVHCTGIRQYVG**GPCKM
C4	EWCGP**EMYQVFLWIQGCLDHRPGMP**GPCKM
C5	EWCGP**CMTCKIQQLLNVPDVAPGCS**GPCKM
C6	EWCGP**WNSWFVLYDGRVKWCMREGG**GPCKM
C7	EWCGP**FADEWPYQRRWFWTIVEVDS**GPCKM
N1	EWCGP**VLDYFWPVWGLCQWHALLMT**GPCKM
N2	EWCGP**RLQAWTLGARGFVILASLFS**GPCKM
N3	EWCGP**WLVAYLSGHTRTWWAPGNFM**GPCKM
N4	EWCGP**TLRWWVYWSGCSNHPFSILS**GPCKM
N5	EWCGP**QKVTSMEINLGFLLGVWSWG**GPCKM

Variable regions of aptamers that break α-factor arrest. Sequence of these variable regions is shown in **bold**, flanked by 5 aa on each side from the thioredoxin platform. The letters C and N refer to aptamers that were isolated from the nonlocalized and nuclear localized libraries, respectively.

*Sph*I-cut pRG2–1 to generate bait plasmids pRG2-Ste50 and pRG2-Ste11. We constructed the low-level prey-expression plasmids pRG3-Ste5 and pRG3-Ste50 by replacing the 2 μ origin in pJG4–5 with a fragment containing a centramere and an autonomously replicating sequence origin from pRS313. Details of constructions are given at www.molsci.org.

We assayed interaction disruption by introducing the low-copy baits pRG2-Ste50, pRG2-Ste11, and plasmids that directed the synthesis of large amounts of peptide aptamers into EGY40, and low-copy prey plasmids pRG3-Ste50, pRG3-Ste5 and pSH18–34 into EGY42. We monitored aptamer-dependent disruption of protein interactions by using the mating interaction assay described. We performed liquid β-galactosidase assays (20) on diploid exconjugants that contained aptamers that caused a distinct diminution in blue color on galactose 5-bromo-4-chloro-3-indolyl-β-D-galactoside plates.

RESULTS

Selection of Aptamers that Confer Resistance to α-Factor. We used two different aptamer-expression libraries; one that directed synthesis of proteins localized to the nucleus and another that directed synthesis of nonlocalized proteins to obtain aptamers that overcame α-factor-induced cell-cycle arrest. We selected aptamers in HCY5, a bar1/bar1, MATa/MATa diploid. Aptamer synthesis was conditionally directed by the Gal1 promoter; under selective conditions, Western gel analysis (data not shown) revealed that $\approx$20,000 aptamers were expressed per cell. From these libraries, we obtained 651 nonlocalized colonies and 151 nuclear colonies that grew in the presence of α-factor. We isolated aptamer plasmids from colonies that displayed galactose-dependent α-factor resistance and reintroduced them into HCY5. Approximately 1 of every 4×10^5 nonlocalized plasmids and 1 of every 1×10^6 nuclear plasmids conferred galactose-dependent resistance to α-factor arrest. Sequencing of 60 aptamer plasmids revealed 20 unique nonlocalized aptamer-variable regions and 18 unique nuclear localized-variable regions. Consistent with previous studies (refs. 6 and 9; unpublished data) in all cases except anti-E2F aptamers (9), these variable regions show no particular similarity to any of the proteins searched in GenBank. We arbitrarily chose 12 peptide aptamers for further study. Their variable region sequences are shown in Table 1.

Mapping Aptamer Function to Neighborhoods Within Pathways. MATa cells respond to α-factor by a mitogen-activated protein kinase-dependent pathway. This pathway branches to cause two separate responses, cell-cycle arrest and induction of genes such as FUS1 (reviewed in ref. 12). To determine whether these aptamers are specific for cell-cycle arrest or whether they act upstream of the branch point, we examined whether they blocked the pheromone induction of FUS1. With the exception of AptC3, all of the aptamers markedly reduced Fus1 induction (47–77% reduction in β-galacosidase activity; data not shown). These results suggest that all aptamers except AtpC3 blocked the pheromone response pathway upstream of the point that cell-cycle arrest and gene induction diverge.

MATa cells can overcome α-factor arrest by derepressing the MATα genes at HMLα, for example by losing the function of the SIR/MAR genes needed for repression (21–23). We tested whether any of these aptamers derepressed HML by determining whether they could overcome α-factor-induced cell-cycle arrest in an HMLa HMRa mutant strain (IH2274). All of the aptamers conferred resistance to α-factor in this strain, indicating that they do not depress HML (data not shown).

Mapping Aptamer Function to Individual Proteins. To map the targets of these aptamers more precisely, we used interaction mating to monitor their interactions with proteins

Table 2. Pheromone response panel

Prey	Gpa1	Ste4	Ste18	Ste20	Ste50	Ste5	Ste7	Ste11	Far1	Ste12	Fus3	Kss1	Cdc28	Cln1	Cln2	Cln3
Bait																
C1	13	—	—	—	—	—	—	402	—	—	—	15	—	—	—	—
C2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
C3	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
C4	—	—	—	—	2,135	—	—	—	—	—	—	—	—	—	—	—
C5	27	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
C6	—	—	—	—	—	794	—	—	—	—	—	—	—	—	—	—
C7	—	—	—	—	37	355	—	—	—	—	—	69	—	—	—	—
N1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
N2	—	—	—	—	—	555	—	—	—	—	—	—	—	—	—	—
N3	—	—	—	—	—	253	—	—	—	—	—	—	—	—	—	—
N5	—	—	—	—	90	—	—	—	—	—	—	—	—	—	—	—
Ste50	—	—	—	—	1,308	395	317	2,220	—	—	—	—	—	—	—	—
Ste7	—	—	—	—	—	844	—	—	—	—	—	—	—	—	—	—
Ste11	—	—	—	—	—	478	—	—	—	—	—	—	—	—	—	—
Fus3	—	—	—	—	—	—	—	85	—	—	—	767	—	—	—	—

Interactions between aptamers and selected pheromone-response proteins. In this experiment, aptamers and Ste50, Ste7, Ste11, and Fus3 are expressed as baits, while potential interacting proteins are expressed as preys. Numbers represent Miller units (35) of β-galactosidase activity determined by liquid assay for interactions that produced blue color on 5-bromo-4-chloro-3-indolyl β-D-galactosidase plates. Activity was corrected by subtracting basal reporter activation by the bait in control strains that express the bait but that contain an empty prey vector (pJG4-5). Standard deviations for all interactions were $<$20%. Gpa1 (36) is the α-subunit G protein; it is also known as Sgc1 (37).

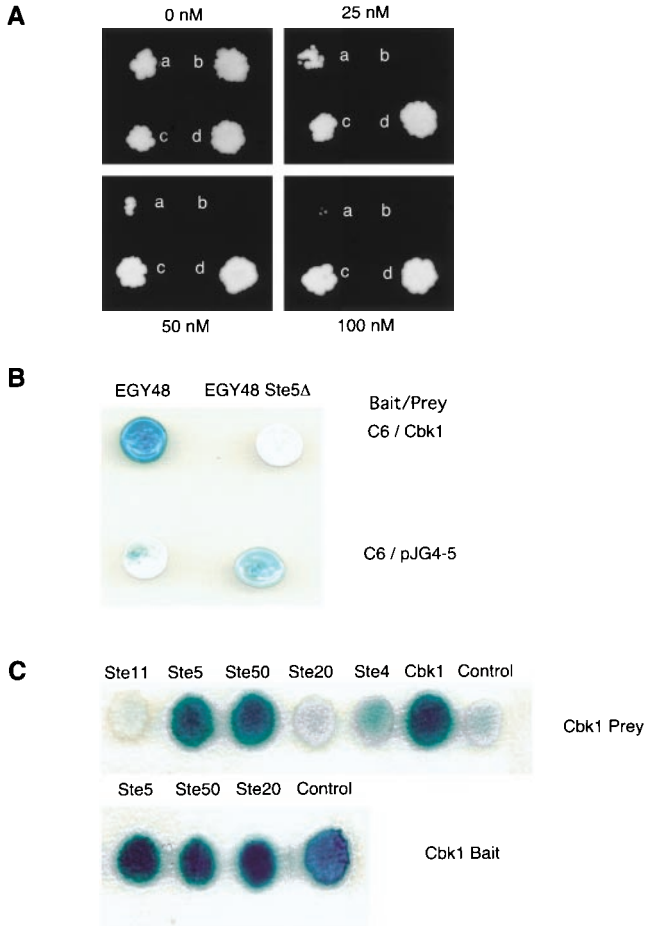

FIG. 1. (*A*) Growth of HCY4 and HCY4 Cbk1Δ in the presence and absence of peptide AptC6. Strains were spotted onto Plates that contained galactose and 0, 25, 50, 100 nM α-factor. (*a*) HCY4 cbk1Δ + pJG4–4. (*b*) HCY4+ pJG4–4. (*c*) HCY4 cbk1Δ + AptC6. (*d*) HCY4 + AptC6. (*B*) Interactions of AptC6 with Cbk1 in strains HCY4 or HCY4ΔSte5. Interactions are scored by activation of the LexAop-lacZ reporter as judged by blue color on 5-bromo-4-chloro-3-indolyl β-D-galactoside plates. *Upper*, experimental. AptC6 is in the bait construct and Cbk1 is in the prey construct. *Lower*, controls. AptC6 is in the bait but pJG4–5, the control plasmid, expresses no prey. AptC6 bait construct gives low-level basal activation and blue color. (*C*) Interaction between Cbk1 and proteins involved in pheromone response. *Upper,* Cbk1 is expressed as a prey, with the indicated proteins expressed as baits. *Lower,* Cbk1 is expressed as a bait and the indicated proteins as preys. Note that, as a bait, Cbk1 directs significant basal transcription. Interactions with Ste5, Ste50, and Ste20 are visible as an increase in blue color above that caused by transcription from the bait.

involved in the pheromone response (Table 2). These experiments revealed three classes of interactions. Six aptamers, C4, C5, C6, N2, N3, and N5, interacted with a single member of the panel, suggesting that these proteins are the aptamer targets. Two aptamers, C1 and C7, interacted strongly with one member of the panel and more weakly with two other members. The interaction patterns of these aptamers are consistent with the idea that the stronger interactors are the targets and that the weaker interactors are caused by the aptamers forming complexes with the primary target, native to yeast, which in turn binds the secondary target. Three aptamers, C2, C3, and N1, did not interact with any panel members.

To identify further potential targets, we used aptamers as baits to find partners in a yeast interaction library. This library (18) is incomplete; for example, PCR analysis (not shown) revealed that the library lacked both Ste50 and Ste5, two targets identified from the panel experiments. We used as baits aptamers C2, C6, N1, N3, and N5, none of which activated transcription of the reporter genes. We identified and characterized their interactors as described by Gyuris *et al.* (14). Of the 16 proteins identified by these experiments (see *Materials and Methods*), none have previously been shown to function in the pheromone-response pathway.

We tested the function of these genes by deleting them and studying the response of the resulting strains to α-factor. One of these genes, CBK1, (cell wall biosynthesis kinase), which was identified by interaction with AptC6, conferred α-factor resistance when deleted. AtpC6 also interacts with Ste5. To determine whether Cbk1, Ste5, or both are the primary targets of AptC6, we first examined whether the cbk1Δ strain showed increased α-factor resistance when AptC6 was expressed. Fig. 1*A* shows that the cbk1Δ strain is more resistant to α-factor in the presence of AptC6. This result suggests that the AptC6 bait may have identified Cbk1 by complexing with native yeast Ste5, which in turn interacted with activation tagged Cbk1 in the interactor hunt. To test this idea, we measured the interaction of AptC6 with Cbk1 in a strain deleted in Ste5. Fig. 1*B* shows that, in this strain, the AptC6/Cbk1 interaction does not occur, showing that Ste5 is required for the AptC6/Cbk1 interaction. We then directly determined whether Cbk1 interacts with Ste5 in interaction mating experiments between Cbk1, Ste5, and other proteins involved in the response to α-factor. Fig. 1*C* shows that Cbk1 interacts with Ste5, Ste50, and Ste20 in interaction mating experiments. Interestingly, Cbk1 is a homolog of *Schizosaccharomyces pombe* Orb6, a protein needed for normal delay of mitosis, and for polarized growth (ref. 26, and see *Discussion*). In fact, in the presence of α-factor, the HCY4 cbk1Δ strain showed a reduced ability to form mating projections (schmoo tips) (B. Nelson and C. Boone, personal communication). These experiments suggest that Cbk1 connects α-factor induced cell-cycle arrest to induced changes in cell morphology (see *Discussion*).

Mapping Aptamer Function to Individual Protein Interactions. Ste5, Ste11, and Ste50 interact with each other in two hybrid experiments (Table 3; refs. 16 and 27–31) suggesting, that they might form a complex with one another (13). We tested the ability of aptamers to disrupt interactions between Ste50–Ste5 and Ste11–Ste50. To do so, we modified the existing interaction trap to facilitate analysis of interaction disruption. We decreased the concentration of bait and prey proteins by lowering the copy number of the bait and prey plasmids. We monitored interaction disruption by diminution of gene expression from a lacZ reporter (Table 3). These experiments indicate that AptC1, which targets Ste11, and AptC4, which targets Ste50, both disrupt the Ste11–Ste50 interaction. By contrast, both AptN3, which targets Ste5, and AptC4, which targets Ste50, disrupt the Ste5–Ste50 interaction. Interestingly, these results (Table 3) suggest that AptN3

Table 3. Protein–protein disruption

Aptamer	Ste50–Ste5	Ste11–Ste50	Interaction Target
C1	69.0 ± 5.2	39.8 ± 6.4	Ste11
C4	47.0 ± 13.3	9.9 ± 2.1	Ste50
N3	41.2 ± 4.8	330.8 ± 37.5	Ste5
pGAH-1	100 ± 9.9	100 ± 1.6	—

Disruption of specific protein–protein interactions. Interactions between Ste50–Ste5 and Ste11–Ste50 were measured as β-galactosidase activity from a LexAop–lacZ reporter. Synthesis of the preys was directed from the vector pGAH-1 (O. Hobert, personal communication; see *Materials and Methods*). For the Ste50–Ste5 interaction, Ste50 was the bait and Ste5 was the prey. For the Ste11–Ste50 interaction, Ste11 was the bait and Ste50 was the prey. Interaction disruption is given as the percentage of β-galactosidase activity in these strains relative to strains in which the aptamers are not expressed. Errors are reported as SD. Note that AptN3, which targets Ste5, disrupts the Ste50–Ste5 interaction but apparently stimulates the Ste50–Ste11 interaction, perhaps by releasing additional Ste11 bait from a complex with Ste5.

Table 4. Deletion analysis

Deletions	C1	C4	N3	Ste11	Ste50	Ste5
Ste11						
1–85						
85–137						
137–738	+++			+	++	++
Ste50						
1–68						
68–118		+++		+++	++	++
118–346		+++			++	++
Ste5						
24–336					+	+
24–586			+++	+++	+++	+
241–336					+	+
336–586			+++	+		
24–143/309–917			+++	+++	+++	+

Interactions between peptide aptamers and portions of Ste5, Ste11, and Ste50. The Ste11, Ste50, and Ste5 fragments (see *Materials and Methods*) are expressed by prey constructs. Aptamers and full-length Ste5, Ste11, and Ste50 are expressed as baits. Strength of the interactions is estimated from the blue color on 5-bromo-4-chloro-3-indolyl β-galactosidase plates. All interactions were scored after 12 hours of growth. +++ indicates intense blue; ++ indicates light blue, and + indicates faint blue; absence of a plus indicates white.

may stimulate the Ste11–Ste50 interaction, perhaps by releasing additional Ste11 from complexes with Ste5.

We then examined whether these aptamers bound sites on their target proteins important for the interactions they disrupted. To do so, we expressed fragments of Ste11, Ste50, and Ste5 in prey constructs and monitored binding of these preys to AptC1, AtpC4, AptN3, Ste11, Ste50, and Ste5 baits. Table 4 shows that AtpC1 binds the same fragment of Ste11 (137–738) as Ste50 and that AptC4 binds the same fragment of Ste50 (68–118) as Ste11. These results suggest that AptC1 and AptC4 disrupt the Ste11–Ste50 interaction by competitively binding to the Ste11–Ste50 interaction surface. Table 4 also shows that AptC4 binds the same fragments of Ste50 (68–118, 118–346) as Ste5 and that AptN3 binds the same fragment of Ste5 (24–586) as Ste50. These results suggest that AptC4 and AptN3 inhibit the Ste5–Ste50 interaction by binding to the interaction surface and inhibiting interaction between the native proteins. Finally, Table 4 shows that AptN3 binds the same fragment of Ste5 (24–586) as Ste11. This result supports the idea that AptN3 releases Ste11 from the Ste11–Ste5 complex, thus stimulating the Ste11–Ste50 interaction observed in our two-hybrid experiments (Table 3).

DISCUSSION

We used peptide aptamers as forward "genetic" agents to identify genes, proteins, and protein interactions that cause a complex phenotype, the arrest of yeast in response to mating pheromone. We isolated aptamers that made yeast resistant to α-factor arrest. We used these aptamers in interaction mating experiments and partial-genome interactor hunts to identify the protein targets of the aptamers that produced the phenotype. We used these aptamers together with a modified interaction trap to identify protein interactions disrupted by these aptamers. These experiments identified known proteins and protein interactions in this pathway and also revealed a function in this pathway for Cbk1, a protein not previously known to affect it.

We identified targets for most of the peptide aptamers by interaction mating experiments against a panel of proteins involved in the α-factor response. Eight of twelve aptamers interacted strongly with a subset of proteins (Ste11, Ste5, and Ste50) involved in the activation of the mitogen-activated protein kinase signaling portion of the response. Ste50 is involved in the transmission of the pheromone signal from $G_{\beta\gamma}$ to Ste11 (30). Ste11 is the mitogen-activated protein kinase kinase kinase (33). Ste5 is the scaffold protein that brings Ste11, Ste7, and Fus3 together (16, 27, 28). Ste5, Ste11, and Ste50 form binary interactions with one another (Table 2; refs. 16 and 27–31), suggesting that they might form a complex with one another (13). The fact that 8 of 12 characterized aptamers target proteins needed to activate the mitogen-activated protein kinase may indicate that protein interactions needed to activate mitogen-activated protein kinase are particularly sensitive to disruption, or alternatively it may reflect selectivity in targeting by thioredoxin peptide aptamers.

The response to α-factor is extremely well studied. Interestingly, however, our experiments identified a new protein that functions in this response and suggested a clue to its function. We identified Cbk1 in interactor hunts that used aptamers as baits with a partial-coverage yeast interaction library. Cbk1 interacted with AptC6. Our results showed that AptC6 bound Ste5, and that Ste5 in turn bound Cbk1, indicating that the Cbk1–AptC6 interaction was because of AptC6 binding to Ste5–Cbk1 complex. Deletion of Cbk1 conferred resistance to α-factor and diminished schmoo formation. The involvement of Cbk1 in the α-factor response makes sense when one considers its *S. pombe* homolog, Orb6. Orb6 is a serine/threonine kinase required for polarized growth and for the proper delay of mitosis (26). These facts led Verde *et al.* (26) to propose that Orb6 coordinates cell morphogenesis (polarized cell growth) and cell cycle (the onset of mitosis).

We suggest that Cbk1 may similarly coordinate cell morphogenesis and cell cycle in *S. cerevisiae.* The following observations are consistent with this idea. First, Cbk1 is required for full sensitivity to α-factor-induced cell-cycle arrest. Second, Cbk1 is required for effective formation of mating projections. Third, Cbk1 interacts physically with Ste20, Ste5, and Ste50, suggesting that it functions in the α-factor response upstream of Ste11. Fourth, the *S. pombe* homolog, Orb6, may interact physically with Orb2, the *pombe* Ste20 homolog (26). Orb6 and Orb2 mutants show synthetic lethality, and Orb6 overexpression partially suppresses Orb2 mutants, suggesting that Orb6 (and Cbk1) acts downstream of Orb2 (and Ste20). These experiments suggest that Cbk1 may act between Ste20 and Ste11, thus connecting Ste20 with other proteins needed for the response to α-factor. In one simple view, Cbk1 may be needed for a normal response to pheromone because it helps Ste20 activate Ste11.

To identify specific protein interactions targeted by these aptamers, we modified the interaction trap. We expressed aptamers at high levels in cells in which we had lowered expression of the bait and prey proteins to accentuate diminution of reporter gene output caused by disruption of the interaction. This tactic allowed us to identify specific disrupted interactions. For example, AptC1 inhibited interaction of Ste50 with Ste11 but not with Ste5, whereas AptN3 inhibited the interaction of Ste50 with Ste5 but not with Ste11. Each aptamer that disrupted an interaction also bound to the portion of its target protein that normally binds the native partner, suggesting that the aptamer competitively inhibited the interaction by binding to the normal interaction interface. It remains possible that other aptamers work by different mechanisms. For example, recent results from our lab (P. Colas, B. Cohen, P. Ferrigno, P. Silver, and R.B., unpublished data) suggest that aptamers that contain nuclear localization sequences could cause phenotypes by binding cytoplasmic targets and sequestering them in the nucleus. This interaction disruption assay should speed characterization of those aptamers that cause phenotypes and thus aid identification of functional connections in genetic networks.

Although identification of targets by using two-hybrid methods is convenient, it should be possible to identify them by using other means, including, for organisms of known ge-

nomes, protein mass spectrometry. When two-hybrid approaches are used, our results illustrate the advantages of identifying targets by mating against ordered yeast arrays (13, 33) rather than by using them as baits in library screens, i.e. (*i*) an ordered array of potential interactors is a fully normalized library; (*ii*) it is easy to detect interactions that result in reporter activation above the basal level caused by the bait alone; and (*iii*) detection of the interaction phenotype does not depend on the interaction enabling the yeast to form a colony on selective medium; as a result, the strength of detected interaction is independent of differences in plating efficiency caused by differential reporter activation (15).

Aptamers targeted to specific proteins bind with K_ds and K_is between 1×10^{-7} and 5×10^{-11} M (refs. 6 and 8–10; P. Colas, B. Cohen, P. Ferrigno, P. Silver, and R.B., unpublished data, and C. W. Xu, Z. Luo, and R.B., unpublished data). From comparison with the reporter transcription levels (6, 15) in the above studies, the affinities of the aptamers isolated here are likely to be in the nanomolar range. This affinity should allow aptamers to inactivate most cellular targets as long as those targets are present at lower concentration. Our results are consistent with those from two previous studies, which show that aptamer targets can be identified with two-hybrid screens. Kolonin and Finley (11) used an anti-Cdc peptide aptamer as a bait to screen a *Drosophila* embryonic cDNA library, and from this screen isolated cyclin-dependent kinases. More recently, Caponigro *et al.* (34) identified two peptide aptamers, displayed from a green fluorescent protein scaffold, that inhibited pheromone response. They used interaction mating against a panel of proteins in this pathway to identify Ste5 as the probable target of one of these aptamers. These studies underscore the utility of peptide aptamers as agents to identify genes involved in processes.

In higher eukaryotes, many phenomena, from senescence to metastasis, are governed by genetic networks whose members and pathway connections are not now understood. Our experiments demonstrate that peptide aptamers are effective dominant-forward "genetic" agents to produce "mutants" in a process and to identify proteins involved in that process. Our results further demonstrate that peptide aptamers can identify specific protein interactions needed for that process. This approach—aptamer-mediated "mutagenesis" followed by identification of proteins and protein interactions targeted by the aptamers—should be particularly useful for analysis of genetic pathways in now-intractable genetic systems, including most plant and animal cells.

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